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Vpr Overcomes Macrophage-Specific Restriction of HIV-1 Env Expression and Virion Production

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SUMMARY

The HIV-1 accessory protein Vpr enhances infection of primary macrophages through unknown mechanisms. Recent studies demonstrated that Vpr interactions with the cellular DCAF1-DDB1-CUL4 E3 ubiquitin ligase complex limit activation of innate immunity and interferon (IFN) induction. We describe a restriction mechanism that targets the HIV-1 envelope protein Env, but is overcome by Vpr and its interaction with DCAF1. This restriction is active in the absence of Vpr in HIV-1-infected primary macrophages and macrophage-epithelial cell heterokaryons, but not epithelial cell lines. HIV-1-infected macrophages lacking Vpr express more *IFN* following infection, target Env for lysosomal degradation, and produce fewer Env-containing virions. Conversely, Vpr expression reduces IFN induction, rescues Env expression, and enhances virion release. Addition of IFN or silencing *DCAF1* reduces the amount of cell-associated Env and virion production in wild-type HIV-1-infected primary macrophages. These findings provide insight into an IFN-stimulated macrophage-specific restriction pathway targeting HIV-1 Env that is counteracted by Vpr.

INTRODUCTION

To establish a persistent infection, lentiviruses encode accessory proteins that are not required for replication in some cell lines, but are necessary for infection in vivo (Malim and Emerman, 2008). Many of these accessory factors have been shown to counteract host restriction factors that can limit HIV-1 infection (Collins and Collins, 2014). Interestingly, transformed cell and primary cell systems vary in the extent to which they express restriction factors targeted by these accessory proteins. For example, primary monocytic cells harbor a postentry block to HIV-1 infection that can be overcome by the simian immunodeficiency virus (SIV) accessory protein Vpx (Berger et al., 2011; Sharova et al., 2008). Vpx binds a substrate adaptor of a cellular ubiquitin ligase complex (damaged DNA binding protein 1-cullin 4-associated factor 1 [DCAF1]) to promote ubiquitylation and proteasomal degradation of cellular restriction factors SAMHD1 (Laguette et al., 2011) and apolipoprotein B-editing complex 3A (APOBEC3A) (Berger et al., 2011). In the absence of Vpx, these restriction factors prevent productive infection of immature monocytic cells.

Despite its importance for infection of immature monocytic cells, no *vpx* gene has been found in any HIV-1 molecular clones and as such, HIV-1 is not able to infect immature monocytic cells that express high levels of SAMHD1 and APOBEC3A. However, Vpr-expressing HIV-1 is able to efficiently infect monocyte-derived macrophages (MDMs) that have lower levels of SAMHD1 and APOBEC3A (Ayinde et al., 2010). Like Vpx, Vpr utilizes DCAF1 and the Rbx1/Cullin4A E3 ubiquitin ligase complex; however, some cellular targets of Vpr have only recently been identified, and their role in facilitating infection of restricted cell types is not well understood. Elegant studies performed in transformed cell-line systems demonstrated that Vpr activates the structure-specific endonuclease (SSE) regulator SLX4 complex through an interaction with DCAF1. Activation of SLX4 leads to evasion of innate immune sensing of viral infection, possibly by enhanced processing of HIV-1 DNA replication intermediates (Laguette et al., 2014). However, the cell lines used for these studies do not require Vpr for infection. Primary MDMs require Vpr for optimal spread, but the mechanism by which Vpr facilitates HIV-1 infection of macrophages has not yet been determined.

We characterized the molecular mechanism by which Vpr enhances HIV-1 infection in primary macrophages using three distinct HIV-1 molecular clones. In contrast to what is observed with Vpx-dependent SIV infection of immature monocytes, we found no effect of Vpr on the first round of infection. However, we noted a striking effect of Vpr on virions produced by infected MDMs, and we noted higher infection rates in subsequent rounds, particularly at low multiplicity of infection (MOI). Surprisingly, Vpr was needed for maximal virion production only when the HIV envelope protein (Env), which is incorporated into virions, was also expressed. Morevover, HIV-1-infected primary MDMs lacking Vpr had markedly reduced amounts of HIV-1 Env protein due to increased lysosomal degradation. MDM-293T heterokaryons similarly restricted Env expression and virion production demonstrating the presence of a dominant restriction in macrophages that can act in *trans*. Based on studies using Vpr mutants

and DCAF1 silencing, DCAF1 was required for Vpr to counteract this macrophage restriction. MDMs lacking Vpr produced more *IFN* RNA upon initial infection, and exogenous IFNα dramatically reduced Env expression and virion production. Thus, innate immune evasion promoted by Vpr impacts HIV-1 spread in macrophages by preventing the activity of a macrophage-specific intrinsic antiviral pathway that targets HIV-1 Env and that interferes with the release of Env-containing virions.

RESULTS

Vpr Is Required for Optimal Spread of HIV-1 in Macrophage Cultures at Low MOI

To explore the mechanism through which Vpr enhances HIV-1 infection of primary MDMs, we constructed a Vpr-null mutant of the 89.6 molecular clone (89.6*vpr*$^-$), which was isolated from the blood of an HIV-1-infected person with AIDS (Collman et al., 1992). As expected based on prior published studies, 89.6*vpr*$^-$ was not defective in permissive cell lines. Virion production in 293T cells transfected with p89.6 or p89.6*vpr*$^-$ proviral DNA plasmids was equivalent over a range of DNA inputs (Figure S1A available online). Additionally, equal mass amounts of 89.6 and 89.6*vpr*$^-$ virus stocks were equally infectious in CEMx174 cells (Figure S1B) as described previously (Balliet et al., 1994). Finally, HIV-1 89.6 and 89.6*vpr*$^-$ were similarly infectious in primary CD4$^+$ T cells (Figure S1C, left).

In contrast, we noted striking differences in virion production by MDMs infected with the same viral stocks of wild-type and mutant viruses, particularly at low inoculum (Figure S1D). The impact of Vpr on virus production was most pronounced when the virus was allowed to spread through the culture for 18 days (up to 20-fold differences; Figure S1C, right three panels). Thus, 89.6*vpr*$^-$ is defective in infection of primary human MDMs, but behaves like wild-type virus in permissive cells such as CEMx174 and 293T cells. These results are similar to those reported by others and confirm that 89.6*vpr*$^-$ behaves as expected (Chen et al., 2004; Connor et al., 1995; Eckstein et al., 2001).

Mechanism of Vpr-Dependent Enhancement of MDM Infection

To determine whether the effect of Vpr we observed on the level of virus measured in the supernatant of infected MDMs resulted from a higher number of initially infected cells or from enhanced spread, we validated an intracellular HIV-1 Gag staining protocol to ensure that we could measure true infection events, rather than cell surface binding or endocytosis of viral particles by MDMs (Figure 1A). At 5 days postinfection (dpi), intracellular Gag staining and flow cytometry revealed a distinct population of cells that expressed HIV-1 Gag in a manner that was completely inhibited by HIV-1 reverse transcription and integration inhibitors azidothymidine (AZT) and raltegravir, respectively (Figure 1A). Thus, we concluded that this assay detected de novo infection of MDMs that was dependent on reverse transcription and integration.

At the earliest time points at which we could detect intracellular Gag (2 dpi), MDMs infected with an equal viral inoculum were equally infected plus or minus Vpr (Figure 1B, left). However by 4 dpi, we observed a higher frequency of infected MDMs with Vpr-containing viruses (2.8-fold, $p < 0.05$; Figure 1B, right). These results were confirmed using a PCR assay that detects HIV-1 DNA (Figures 1C and S2A); Vpr did not stimulate the amount of cell-associated provirus detected at 2 dpi, but by 4 dpi, we detected 3- to 4-fold more provirus in Vpr-expressing HIV-infected MDMs (Figures 1C and S2A).

In MDMs, reverse transcription and integration typically require 2–3 days for completion of the first round of infection (Spivak et al., 2011). Thus, at 4–5 dpi, the first round of replication has been completed, and the second round of infection has begun. Therefore, to distinguish initial infection from spread, we inhibited subsequent rounds of infection by the addition of raltegravir to a subset of MDMs at 2 dpi. We then harvested all the cells on day 4. In the absence of raltegravir, we again observed a 2.6-fold increase in the frequency of infected cells with Vpr-containing viruses (Figure 1D). However, in a side-by-side experiment using cells from the same donor, the addition of raltegravir abrogated this difference (Figure 1D). Thus, under the conditions of our assay, Vpr did not affect the initial infection of MDMs and primarily acted by stimulating spread of virus to new target cells.

Vpr Facilitates Spread in Macrophages by Increasing Virion Production

To better understand how Vpr promotes spreading infection of MDM cultures, we sought to distinguish effects of Vpr on different HIV-1 replication stages. We hypothesized that under conditions in which Vpr does not increase infection in the first round of HIV-1 replication (Figure 1D), it may affect the number of virions produced per infected cell. To examine this, we blocked spread of HIV-1 at 2 dpi using a concentration of raltegravir sufficient to fully inhibit new HIV-1 infection events and measured virion production under these conditions (Figures 1A and 1D). We found that Vpr increased virion production by MDMs infected with a Vpr-containing virus an average of 5-fold compared to HIV-1-infected MDMs lacking Vpr, which was statistically significant across multiple donors (Figure 2A). Of note, there were no significant differences in infected cell number that could explain these large differences in virion production (Figures 2B and S3A–S3E).

We also observed dramatic effects of Vpr on virion production in MDM cultures in which HIV-1 was allowed to spread to saturation over 20 days, equalizing the infection rates based on flow cytometry (Figures 2C and 2D) and Gag DNA (Figures 2E and S2B). Under these conditions of equivalent infection, Vpr increased virion production an average of 5-fold ($p < 0.01$; Figure 2F).

Similar results were observed when MDMs were infected with a T cell-tropic HIV-1 pseudotyped with a macrophage-tropic Env (NL4-3 pseudotyped with YU-2 Env). This virus is active for a single round of infection, but cannot spread in MDM cultures. Again, we observed that Vpr did not significantly modulate the number of infected MDMs (Figure S4A), but significantly increased the number of virions released by MDMs infected with wild-type virus (Figures S4B and 2G). The more modest phenotype observed in the single-round system is likely due to the use of a higher inoculum, which results in a reduced requirement for Vpr (Figure S1D), or to other differences between the NL4-3 and 89.6 viruses.

Vpr Counteracts MDM-Specific Interference with Release of Env-Containing Virions

Importantly, using the single-cycle infection system described above, we noted that Vpr did not alter the release of virions

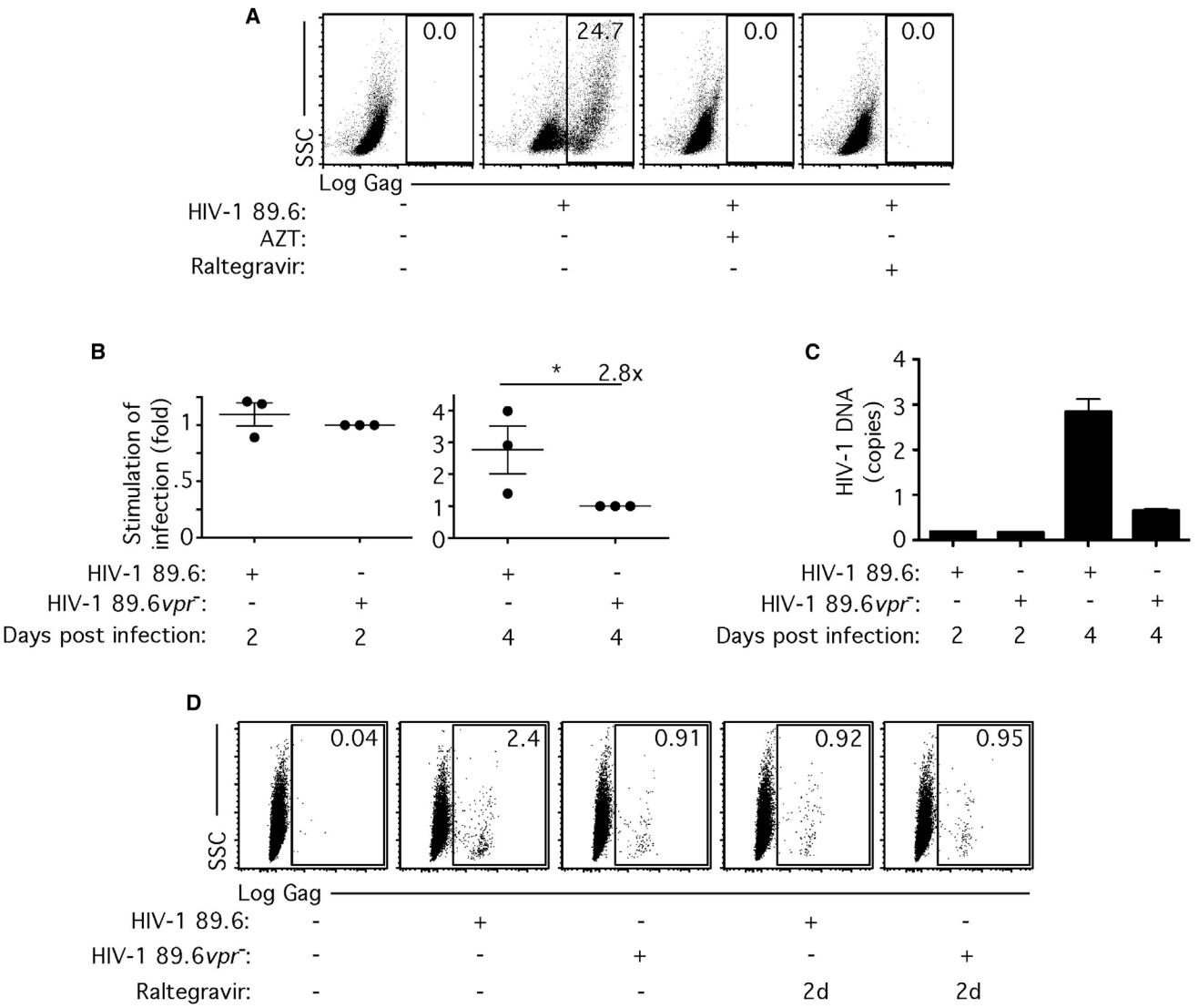


Figure 1. Vpr Does Not Increase the First Round of Infection in Primary Human MDM Cultures

(A) Flow cytometric analysis detecting bona fide infection of MDMs by intracellular HIV-1 Gag p24 stain. Cells were infected for 5 days with 50 μg of HIV-1 89.6 and treated with 20 μM azidothymidine (AZT) or 2 μM raltegravir where indicated.

(B) Summary graph showing the fold difference in infection frequency observed in MDMs treated with 1 μg of wild-type or Vpr mutant virus. By definition, the mutant virus normalizes to 1.0.

(C) qPCR analysis of *gag* DNA in MDMs infected with the indicated viruses. Levels of *gag* DNA were normalized for *β-actin* (*ACTB*) DNA levels to account for differences in cell number (Figure S2A). Each bar represents the average of three replicates from the same donor. Error bars represent SEM. *$p < 0.05$, two-tailed paired t test.

(D) Flow cytometric analysis of intracellular HIV-1 Gag p24 expression in MDMs infected with 1 μg of the indicated viruses and analyzed at 4 dpi. Cells were treated with 2 μM raltegravir at 2 dpi where indicated. See also Figures S1 and S2.

from MDMs infected with a mutant virus that did not express Env from the integrated provirus (NL4-3-ΔE-EGFP, pseudotyped with Env^{YU-2}) (Figure 2G). To confirm these results, we also examined virion production by MDMs infected with 89.6 Env-null and Env/Vpr-null mutants relative to wild-type and Vpr-null viruses. We again found that mutation of Env eliminated any significant effect of Vpr on virion production (Figure 2A). In contrast, Env and Vpr did not affect the release of virions by permissive cell lines (transfected 293T cells; Figure S1A). Because Env is incorporated into virions, these data support a model in which Vpr counteracts an MDM-specific factor or pathway that interferes with the release of Env-containing virions.

Vpr Counteracts Interference with Env Expression by HIV-Infected MDMs

Because our data strongly suggest that Vpr counters an MDM-specific factor that targets Env-containing virons, we asked whether MDMs also interfered with expression of cell-associated Env in the absence of Vpr. Indeed, we observed that the amount of HIV-1 Env protein detected in MDMs was Vpr

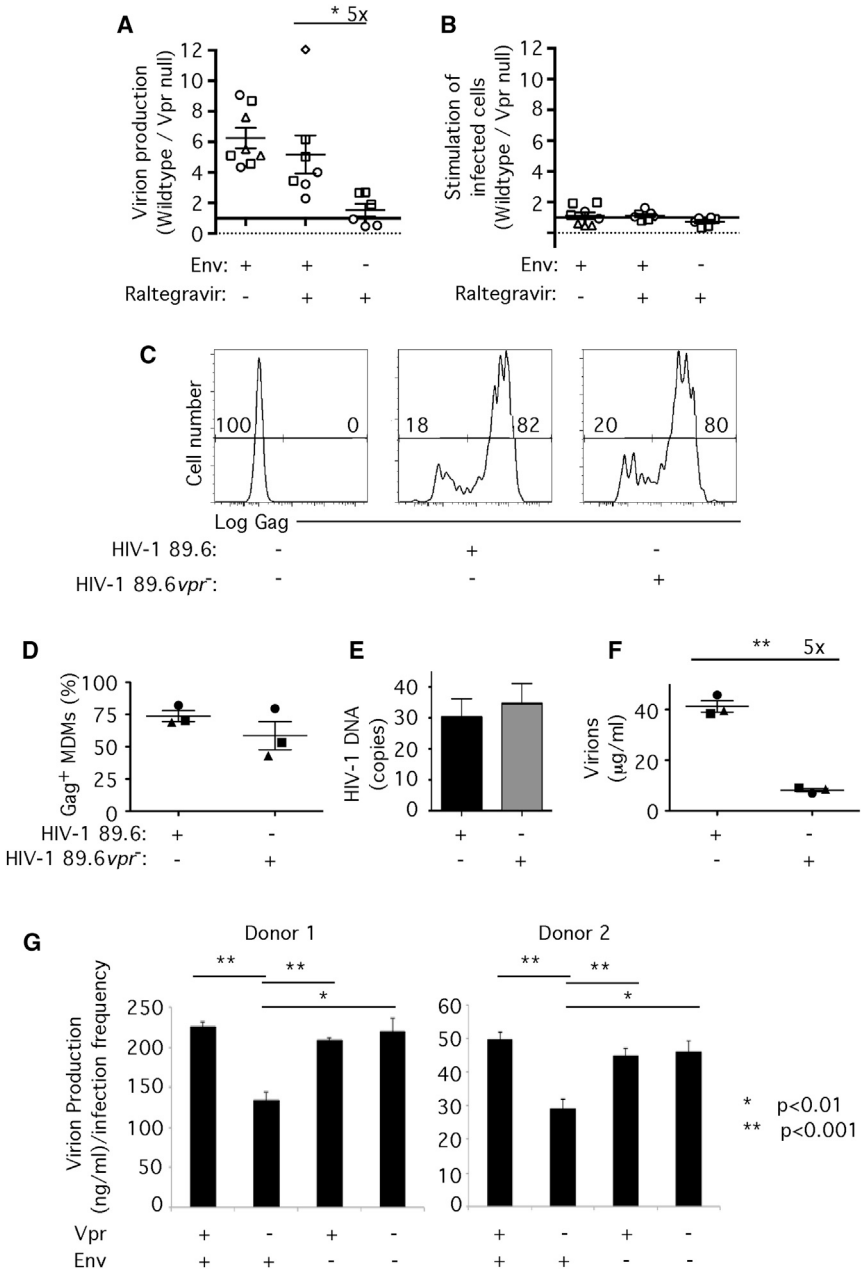


Figure 2. Vpr Increases Virion Production in HIV-1-Infected Primary MDMs

(A) Summary graph of the effect of Vpr on virion production, calculated as the fold change in virion production by MDMs infected by wild-type 89.6 relative to 89.6*vpr*$^-$. Virion production was normalized for the absolute number of Gag$^+$ cells acquired by flow cytometry within a fixed interval of time. See also Figure S3.

(B) Summary graph of the effect of Vpr on the number of infected cells, calculated as the fold change in the number of MDMs infected by wild-type 89.6 relative to 89.6*vpr*$^-$. Each shape in (A) and (B) represents a replicate from one experiment from a single donor. Error bars represent SEM.

(C) Flow cytometric analysis of HIV-1 Gag p24 expression in MDMs infected for 20 days with 1 µg of the indicated viruses.

(D) Compilation of flow cytometry experiments staining for intracellular HIV-1 Gag p24 in MDMs infected as in (C).

(E) qPCR analysis of *gag* levels in MDMs from a duplicate well from the experiment in (C). Levels of *gag* DNA were normalized for *β-actin* (*ACTB*) DNA levels to account for differences in cell number. See also Figure S2.

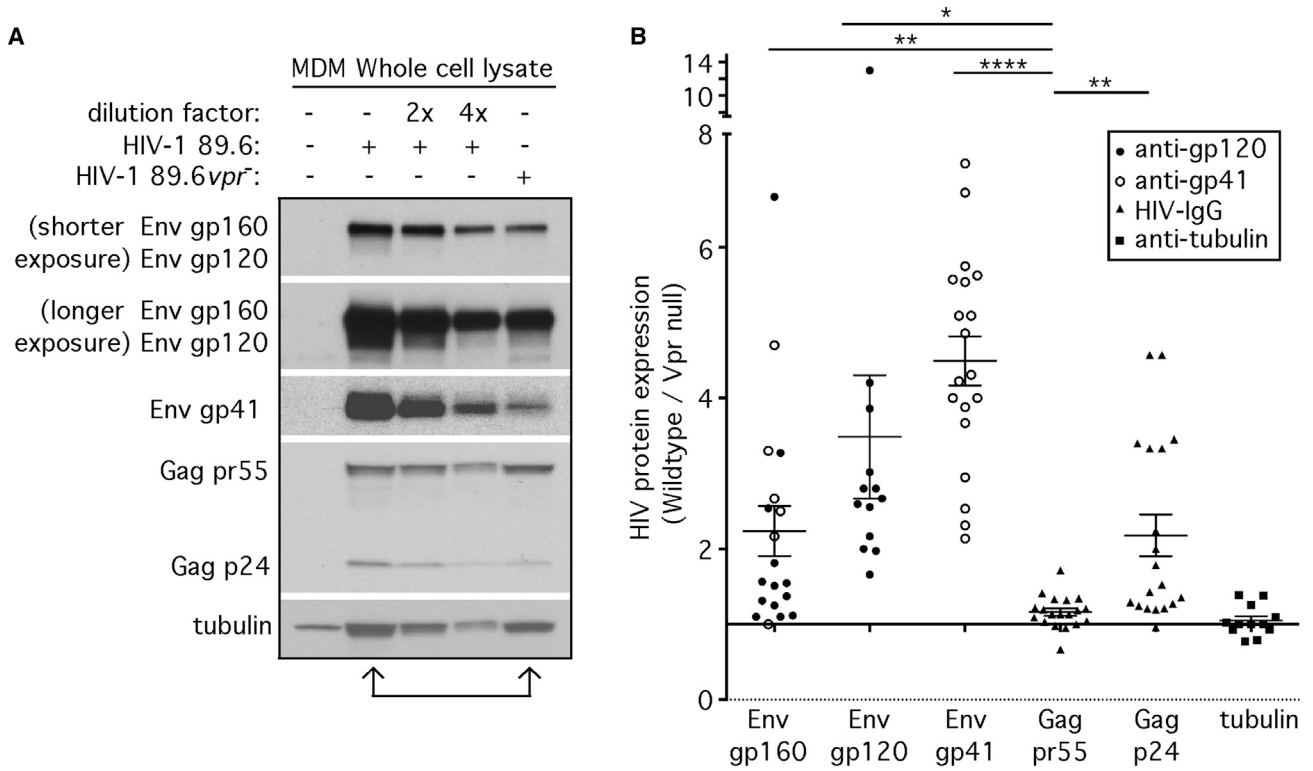
(F) Virion production based on HIV-1 Gag p24 levels in the supernatant of MDMs from three donors for which infection rates were shown in (D). Error bars represent SEM. *p < 0.05, **p < 0.01, two-tailed paired t test. In (D) and (F) each symbol represents a different donor (n = 3).

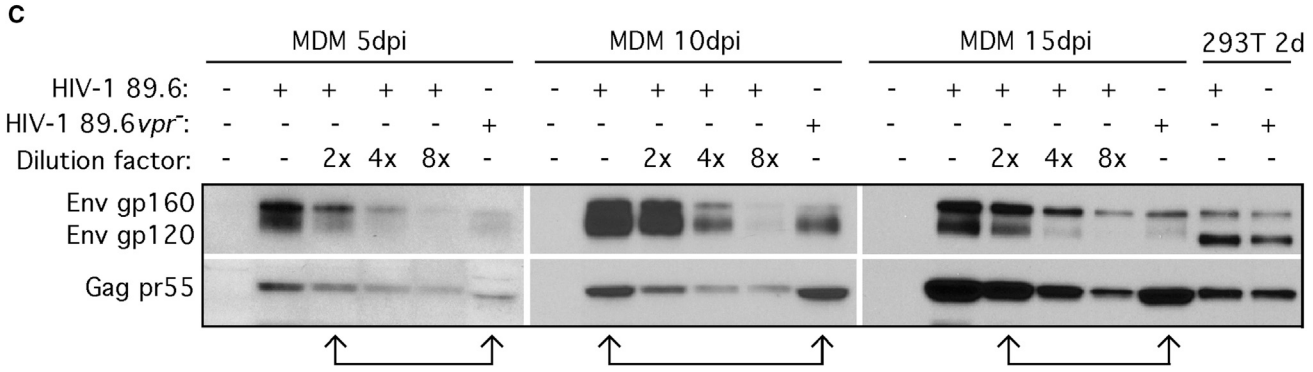
(G) Summary graph of virion production by MDMs 4 days postinfection with YU-2 envelope-pseudotyped NL4-3 wild-type or containing the indicated mutations. The mean ratio of viral production/infection frequency is shown ± SD. *p < 0.01, **p < 0.001, two-tailed paired t test, n = 3 replicates from each donor. See also Figure S4.

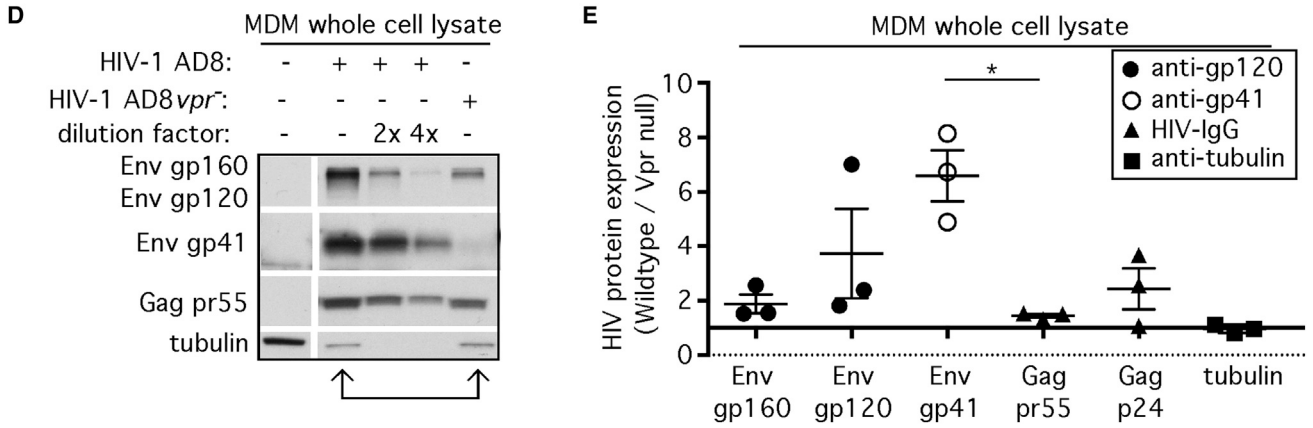
dependent, whereas the level of Gag precursor (pr55) was not (Figures 3A and 3B). The effect of Vpr on Env was MDM-specific because we observed no significant effect of Vpr on Env expression in 293T cell lines transfected with proviral DNA (Figure S5A). To more accurately assess the effect of Vpr on Env per infected cell, we examined serial dilutions of whole-cell lysate (WCL) and compared Env levels only for dilutions in which Gag pr55 expression was matched. Vpr consistently increased the expression of the processed gp41 form of Env (4- to 5-fold; Figures 3A and 3B). The gp160 precursor and processed gp120 forms of Env were also affected, but to a lesser extent than gp41 (2- to 4-fold; Figures 3A and 3B). Vpr also significantly increased the amount of processed Gag p24 in most donors (Figures 3A and 3B). However, this effect was more modest on average (about 2-fold) and varied considerably across donors.

To determine whether modulation of Env expression varied over the time course of infection, we analyzed Env expression in MDMs over time. To accurately compare the level of Env, we again performed serial dilutions and compared samples in which Gag pr55 expression was matched. We found that Vpr enhanced Env gp160 and gp120 expression even at 5 dpi (Figure 3C). However, the effect of Vpr on these forms of Env became more dramatic over time (Figure 3C). Indeed, by 20 dpi, Vpr increased Env gp160 and gp120 7- and 15-fold, respectively (Figures S5B and S5C).

To verify that these effects of Vpr were not specific to a single molecular clone of HIV-1, we performed similar experiments using HIV-1 molecular clone AD8 and a well-characterized AD8 Vpr-null mutant (Rey et al., 1998; Theodore et al., 1996). We again observed no defect in Env expression by the mutant virus in transfected 293T cells (Figure S5D). However, MDMs infected with AD8*vpr*$^-$ displayed substantially lower levels of Env relative







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to Gag pr55 than MDMs infected with wild-type AD8 (Figure 3D). As with 89.6, this effect of Vpr was especially notable for Env gp41 where we observed an average of 7-fold more protein in MDMs infected with wild-type virus (Figures 3D and 3E). Again, we observed a more variable effect of Vpr on Env gp160 precursor (Figures 3D and 3E). Furthermore, we also observed effects of Vpr on NL4-3 Env when YU-2 Env-pseudotyped NL4-3 was used to infect MDMs (Figure S4C). Thus, the ability of Vpr to increase Env protein expression and virion production in MDMs is conserved among HIV-1 variants isolated from different HIV-1-infected people.

To determine whether the defect in cell-associated Env expression also led to diminished incorporation of Env into virions, we lysed virus from infected MDM supernatants and analyzed it by immunoblot. We found that Vpr significantly enhanced Env incorporation into virions by an average of 2- to 3-fold (Figures S5E and S5F). However, when MDMs were infected with equal mass amounts of MDM-derived virus containing or lacking Vpr, we observed equal numbers of infected cells (data not shown). Thus, MDMs express a factor or pathway that interferes with incorporation of Env into virions and that inhibits their release, but under the conditions of our assay, the magnitude of the interference was not sufficient to alter the infectivity of the residual recovered virions.

Vpr Prevents Lysosomal Degradation of HIV-1 Env in MDMs

To determine the mechanism by which MDMs interfere with Env expression, we performed a pulse-chase analysis of Env protein synthesis and decay in infected MDMs at 10 dpi. Vpr did not affect the quantity of the precursor form of Env (gp160) synthesized within the 1 hr pulse (n = 8; Figure 4A; data not shown). However, Vpr increased the half-life of the processed form (gp120) from 3.3 hr to 7.7 hr ($p < 0.0001$; Figures 4A and 4B). In contrast, there was no significant effect of Vpr on the half-life of HIV-1 Gag pr55 or Gag p24 (Figure 4A; data not shown). Based on quantitation of *β-actin* and HIV-1 *gag* DNA, the numbers of infected cells added to the assay were similar (Figures 4C and 4D). In sum, these results indicate that Vpr increases Env protein expression by a posttranslational mechanism that prevents Env degradation.

To determine which degradative pathways affected Env in MDMs infected with 89.6*vpr*$^-$, we treated cells that were pulse labeled for 1 hr and chased with nonlabeled media for 8 hr with ammonium chloride, an inhibitor of lysosomal degradation, or MG132, an inhibitor of proteasomal degradation. Ammonium chloride, but not MG132, partially rescued Env gp120 expression ($p < 0.002$; Figures 4E and 4F). In contrast, ammonium chloride treatment did not significantly affect Gag pr55 levels or Gag p24 over the 8 hr time course of this assay (Figures 4E and 4G). These data demonstrate that HIV-1 Env is targeted to lysosomes in HIV-1-infected cells lacking Vpr.

Vpr Counteracts a Dominant Restriction of Env and Virion Production in MDMs

To determine whether Env expression is diminished in MDMs because of a negative restriction factor that is counteracted by Vpr or because MDMs lack a positive cofactor that Vpr provides, we examined Env expression and virion production in heterokaryons of restricted (MDM) and permissive (293T) cells. Heterokaryons were generated using Newcastle disease virus (NDV) fusion proteins (F and HN) and HIV-1, which promotes cellular fusion via Env on transfected 293T cells and CD4 on MDMs. In these experiments, 293T cells were transfected equally by HIV-1 89.6 and 89.6*vpr*$^-$, and spread of HIV-1 to MDMs was prevented by the addition of raltegravir (Figures 5A and 5B). Similar to previously published work (Kaushik et al., 2009; Sharova et al., 2008) we observed that NDV fusion proteins and HIV-1 enhanced the fusion of transfected 293T cells to uninfected MDMs, and fewer than 10% of 293T cells were unfused at the time of harvest (Figure 5C). Remarkably, we found that MDM-293T heterokaryons restricted Env expression and virion production, whereas 293T homokaryons did not (Figures 5D and 5E). Moreover, the heterokaryon-specific restriction was counteracted by Vpr (Figures 5D and 5E). Collectively, our data indicate that Vpr counteracts a macrophage-specific restriction factor that targets Env for lysosomal degradation and impairs the release of virions.

DCAF1 Is Required for Vpr to Counteract Restriction of Env Expression and Virion Production in MDMs

Studies performed in permissive cells have shown that HIV-1 Vpr interacts with a cellular ubiquitin ligase complex through the adaptor protein DCAF1. Because lentiviral accessory proteins commonly utilize ubiquitin ligase substrate adaptors to target restriction factors for degradation, we examined whether DCAF1 is required for Vpr to overcome the restriction we observed in MDMs. Interestingly, we observed that a mutant Vpr (Vpr^{Q65R}) that is defective at DCAF1 interactions, DCAF1-dependent cell

Figure 3. HIV-1 Vpr Increases Env Expression in MDMs

(A) Immunoblot analysis of HIV-1 protein expression in whole-cell lysates (WCLs) from MDMs infected for 10 days with 1 μg of the indicated viruses. Env gp160 and gp120 were detected with anti-gp120 antibody, gp41 was detected by anti-gp41 antibody, and Gag pr55 and p24 were detected with HIV-Ig. WCLs from MDMs infected with wild-type 89.6 were serially diluted as indicated. Arrows denote samples containing comparable levels of Gag pr55 in the presence or absence of *vpr*.

(B) Summary graph quantifying the fold increase in expression of the indicated HIV-1 proteins in MDMs infected with wild-type 89.6 relative to 89.6*vpr*$^-$ as in (A). Each symbol denotes the antibody used to detect the indicated protein and represents a separate experiment performed in a different donor (n = 19). Error bars represent SEM. *$p < 0.05$, **$p < 0.005$, ****$p < 0.00005$, two-tailed paired t test.

(C) Immunoblot analysis of viral protein levels in WCLs from MDMs infected for the times indicated with 1 μg of the indicated viruses and from 293T cells transfected for 2 days with the indicated HIV-1 genomic DNA plasmids. WCLs from MDMs infected with wild-type 89.6 were serially diluted as indicated.

(D) Immunoblot analysis of viral protein levels in WCLs from MDMs infected for 6 days with 1 μg of wild-type AD8 or AD8*vpr*$^-$. Lysates from MDMs infected by wild-type AD8 were serially diluted in loading buffer as indicated.

(E) Summary graph of the effect of Vpr on expression of the indicated HIV-1 proteins, calculated as fold change in protein levels in MDMs infected for 6 days with 1 μg of wild-type AD8 relative to AD8*vpr*$^-$. Each symbol denotes the antibody used to detect the indicated protein and represents a separate experiment performed in a different donor (n = 3). Error bars represent SEM. *$p < 0.05$, two-tailed paired t test. See also Figures S4C and S5.

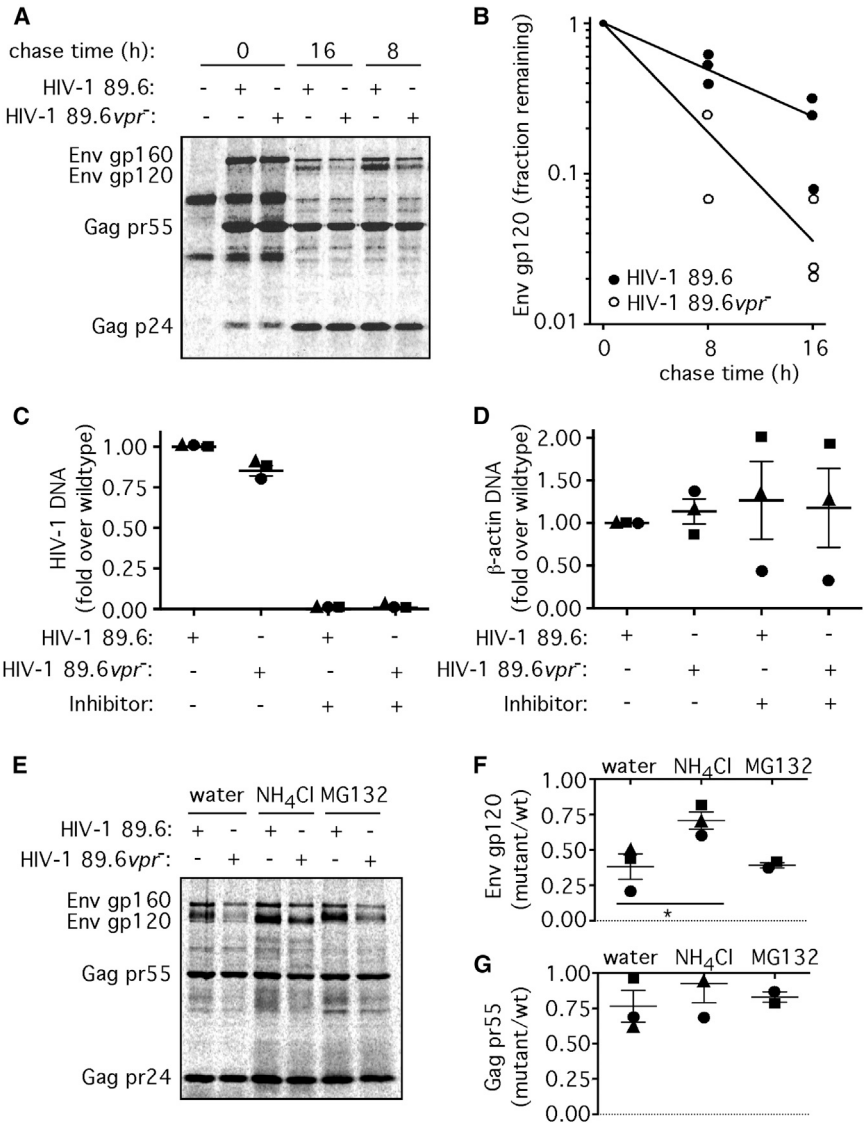

Figure 4. Vpr Prevents Lysosomal Degradation of Env in Primary Human MDMs

(A) Radioimmunoprecipitation assay of HIV-1 proteins from primary human MDMs infected with 1 μg of wild-type 89.6, cultured for 10 days prior, and metabolically labeled with [^{35}S]Met/Cys for 1 hr. The labeled cells were chased for the indicated time periods, immunoprecipitated, and subjected to SDS-PAGE.

(B) Summary of quantified data from the experiments performed as in (A). The fraction of gp120 remaining at 8 hr or 16 hr relative to T0 was calculated as described in Supplemental Experimental Procedures. Each dot represents a different donor from a separate experiment (n = 3). Best-fit curves were obtained by nonlinear regression analysis.

(C and D) qPCR analysis of *gag* (C) and *β-actin* (D) DNA levels in cells analyzed in (B). Cells were treated with 10 μg/ml AMD3100 and 20 μM maraviroc or 2 μM raltegravir during infection where indicated. Each shape represents a different donor from a separate experiment (n = 3).

(E) Radioimmunoprecipitation assay of HIV-1 proteins from primary human MDMs infected and radiolabeled as described for (A). As indicated, labeled cells were chased for 8 hr with or without inhibitors of lysosomal and proteasomal degradation (20 μM NH_4Cl and 2.5 μM MG132, respectively).

(F and G) Quantification of Env gp120 (F) and Gag pr55 (G) levels in cells treated as in (E). Env expression was calculated as the fold change in protein measured in WCLs from MDMs infected by 89.6*vpr*$^-$ relative to wild-type 89.6. Each shape represents a different donor from a separate experiment (n = 3). Error bars represent SEM. *$p < 0.05$, two-tailed paired t test.

cycle arrest, and SLX4 complex activation (DeHart et al., 2007; Hrecka et al., 2007; Laguette et al., 2014) was similarly defective at enhancing Env expression and virion production (Figure S6).

To more directly demonstrate a causal relationship between DCAF1-Vpr interaction and Vpr-mediated increases in virion production and Env expression, we silenced *DCAF1* expression in primary human MDMs. To accomplish this, we used an shRNA-expressing lentiviral construct that had been optimized to maximize silencing while limiting antiviral responses in MDMs (Pertel et al., 2011). Using this system we reproducibly achieved efficient silencing of *DCAF1* expression (Figure 6A). Remarkably, we observed that *DCAF1* silencing dramatically reduced Env expression (Figure 6B) and virion production (Figure 6C) in wild-type HIV-1-infected MDMs. Indeed, without DCAF1 expression, Env expression was similar between wild-type and Vpr-null viruses (Figure 6B). Thus, these studies indicate that Vpr requires interaction with the DCAF1-DDB1-CUL4 E3 ubiquitin ligase to overcome restriction of virion production and Env expression in HIV-1-infected MDMs.

Vpr Disrupts a Pathway that Leads to Induction of Type I IFN

Recent studies have shown that inducible Vpr expression in a HeLa cell line activates the SSE regulator SLX4 complex (Laguette et al., 2014). In this cell-line system, induction of Vpr increases polo-like kinase-1 (PLK-1) levels and stimulates the ubiquitylation and turnover of MUS81-EME1 endonucleases (Laguette et al., 2014) in a manner that requires the DCAF1-DDB1-CUL4 E3 ubiquitin ligase complex. We demonstrate here that this pathway is intact in primary human MDMs and CD4$^+$ lymphocytes (Figures S7A and S7B). Compared to infected cells lacking Vpr, wild-type HIV-1 infection led to the accumulation of PLK-1 and increased the turnover of MUS81 and DCAF1 in primary MDMs (Figure S7A). In primary CD4$^+$ T cells, we additionally noted decreased amounts of the Vpr-interacting protein uracil-DNA glycosylase 2 (UNG2) as previously reported (Priet et al., 2003; Wen et al., 2012). In MDMs, the levels of UNG2 were not assayable due to lower expression levels (data not shown). Interestingly, DCAF1 was diminished as early as 5 hr postinfection (hpi) in MDMs, consistent with its utilization by Vpr (Figure S7C). Vpr-dependent downmodulation of DCAF1 at 5 hpi was completely reversed by the addition of the proteasome

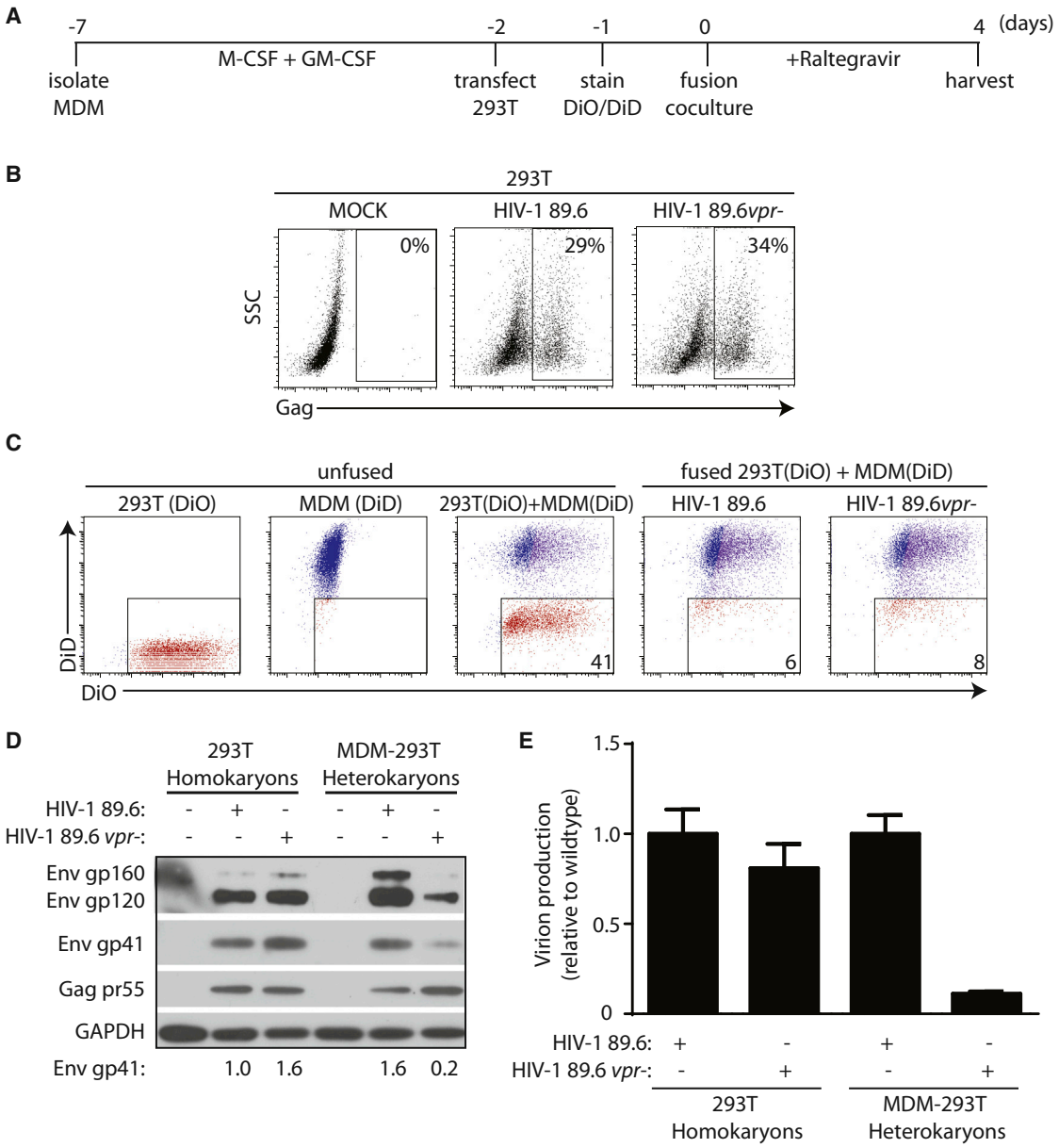

Figure 5. Vpr Counteracts a Dominant Restriction of Env and Virion Production in MDMs

(A) Outline of experimental setup in which 293T cells transfected with HIV-1 and paramyxovirus fusion proteins were fused with uninfected MDMs for 4 days in the presence of raltegravir to prevent viral spread to unfused MDMs.

(B) Flow cytometric analysis of intracellular Gag expression by 293T at 2 days posttransfection with the indicated HIV expression plasmid.

(C) Flow cytometric analysis of 293T cells that were mock transfected (unfused) or transfected with paramyxovirus fusion proteins and the indicated HIV plasmids (fused), stained with DiO, and cultured as indicated with DiD-stained MDMs in the presence of raltegravir. Numbers represent frequency of residual unfused 293T cells.

(D) Immunoblot of 293T cell homokaryons and MDM+293T heterokaryons. Env gp41 quantitation reflects densitometrically determined values relative to Gag pr55.

(E) Virion production by 293T cell homokaryons and MDM-293T heterokaryons measured and calculated as described in Figure S3 and normalized to wild-type. Error bars represent SEM.

inhibitor MG132 (Figure S7C), indicating that virion-associated Vpr utilizes DCAF1 at very early time points prior to the establishment of productive infection.

Because activation of the DCAF1-SLX4 pathway diminishes viral sensing and limits type I IFN responses in HeLa cells (Laguette et al., 2014), we examined IFN responses in infected MDMs. Remarkably, we found that wild-type HIV-1-infected MDMs exhibited lower *IFNA1* gene expression compared to Vpr-null-infected MDMs at 12 hpi (Figure 7A). Furthermore, the magnitude of the difference in *IFNA1* response correlated with

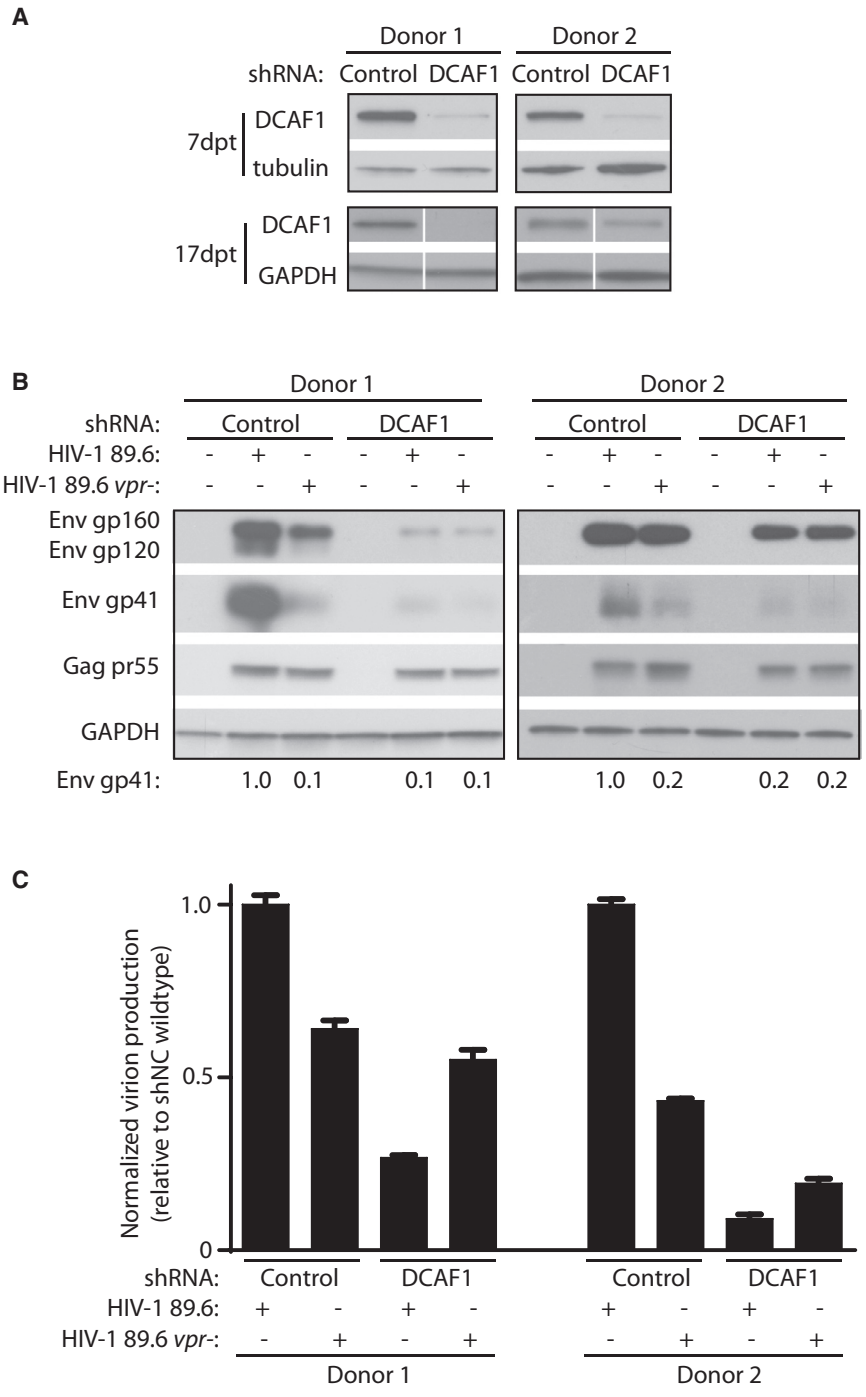

Figure 6. DCAF1 Is Required for Vpr to Increase Virion Production and Env Expression

(A) Immunoblot analysis of WCLs prepared from MDMs transduced with lentiviral vectors expressing shRNAs targeting DCAF1 or luciferase (Control) and incubated for 7 days (first 3 days with puromycin) (Pertel et al., 2011). dpt, days post-transduction.

(B) Immunoblot analysis of WCLs from MDMs treated as described in (A) and then infected with 1 μg of wild-type 89.6 or 89.6*vpr*⁻ for 10 days. Quantitation reflects densitometric analysis of Env gp41 expression normalized for Gag pr55 expression.

(C) Normalized virion production by MDMs treated and infected as described in (B). Error bars represent SEM. See also Figure S6.

the degree to which Vpr enhanced virion production at 10 dpi (Figure 7B).

To directly assess whether type I IFN may contribute to restriction of Env and virion production in MDMs, we treated infected MDMs with IFNα at 7 dpi and harvested at 10 dpi (Figure 7C). Because we observed that IFNα completely inhibited new infections when added at day 0, we added raltegravir to established infections at the time of IFNα addition to eliminate confounding effects due to differences in spread. Under these conditions, we observed that IFNα treatment strikingly reduced HIV-1 Env gp41, gp120, and gp160 protein levels relative to Gag pr55 in multiple donors (Figure 7D). Additionally, IFNα treatment suppressed virion production per infected cell (Figure 7E). These data support a model in which Vpr facilitates Env expression and release of Env-containing virions by limiting induction of IFNα production upon HIV-1 infection of MDMs.

DISCUSSION

Vpr is an HIV-1 accessory protein and virulence factor associated with high viral loads and progression to AIDS (Lang et al., 1993; Somasundaran et al., 2002). In vitro, Vpr promotes HIV-1 infection of restricted primary cells such as macrophages (Le Rouzic and Benichou, 2005). However, the mechanism by which Vpr facilitates infection is poorly understood.

In agreement with other studies, we observed that Vpr enhances viral infection and spread in macrophages (Chen et al., 2004; Connor et al., 1995; Eckstein et al., 2001). Here we demonstrate that Vpr enhanced spread by increasing the number of virions released per infected cell when HIV-1 Env was also expressed. In addition, all Vprs tested (89.6, NL4-3, and AD8) dramatically increased the expression of HIV-1 Env, a necessary component of HIV-1 virions that binds to the CD4 receptor and coreceptors (Freed and Martin, 1995) to facilitate viral fusion to target cells. Env is also necessary for formation of the virological synapse, which is required for cell-to-cell spread (Jolly et al., 2004). Therefore, it is likely that Vpr-dependent increases in Env expression also enhance cell-to-cell spread by facilitating virological synapse formation.

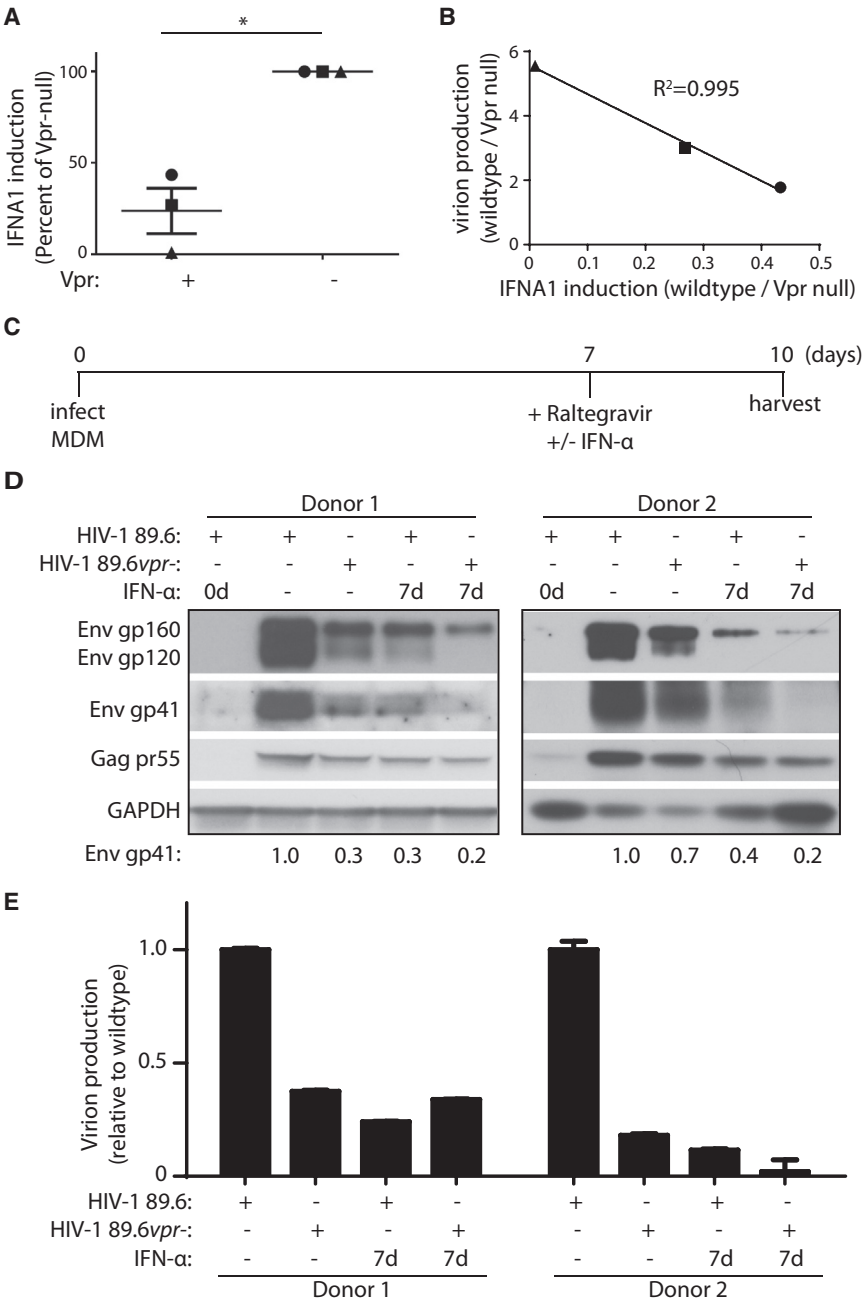

Figure 7. Vpr Counteracts an Antiviral Interferon Response in MDMs that Restricts Env and Virion Production

(A) *IFNA1* gene induction in MDMs infected for 12 hr with 100 μg of wild-type 89.6 or 89.6*vpr*⁻ as measured by qRT-PCR, normalized to *ACTB*, and expressed as fold induction relative to mock and normalized to Vpr null (n = 3 donors).

(B) Graphical analysis demonstrating an inverse correlation between *IFNA1* gene induction as measured in (A) and Vpr stimulation of virion production at 10 days postinfection with 1 μg of each virus. Best-fit curve and R^2 value were obtained by linear regression analysis (n = 3 donors).

(C–E) (C) Outline of experimental setup for MDMs treated with 500 U/mL recombinant human IFNα and assessed for Env expression (D) and virion production (E) in two independent experiments. Error bars represent SEM. *p < 0.05, two-tailed paired t test. See also Figure S7.

Indeed, we observed a greater effect of Vpr when HIV-1 was allowed to spread through the MDM culture.

The results presented herein demonstrate that macrophages express a cell type-specific restriction factor that targets newly made Env protein and Env-containing virions. This determination is strongly supported by several key observations: (1) Vpr enhanced virion production only when Env was expressed (null mutations in 89.6 and NL4-3 *env* eliminated the ability of Vpr to stimulate virion production), (2) Vpr stimulated Env expression and Env-containing virion production in restricted cells (macrophages), but not in permissive cell lines (293T), (3) permissive cells acquired the capacity to restrict Env expression and release of Env-containing viral particles when fused to restricted cells (primary macrophages), indicating that macrophages harbor a dominant restriction factor that can act in *trans*, and (4) Vpr counteracts the restriction in macrophages and in 293T-macrophage heterokaryons. In sum, these data demonstrate that in the absence of Vpr, a dominant factor restricts the expression of Env protein and the release of Env-containing virions in macrophages. Moreover, Vpr functions to counteract this restriction.

While our data clearly demonstrate that restriction of cell-associated Env occurs via accelerated lysosomal degradation in MDMs lacking Vpr, additional studies are needed to understand the fate of Env-containing virions in these cells. Prior studies have shown that cellular proteins that bind Env can also reduce release of Env-containing virions (Ross et al., 1999; Lama et al.,1999). Thus, Vpr could enhance virion release by counteracting an MDM-specific factor that binds Env on budding virions, reducing virion release. Based on pulse-chase analysis of cell-associated p24 summarized herein, Vpr did not detectably affect virion assembly, budding, retention, and degradation up to 16 hr postsynthesis. However, if MDMs both retain and degrade virions in the absence of Vpr, virion retention could mask virion degradation at early time points. Based on western blot analysis, which provides an assessment of steady state cell-associated p24 averaged over longer time periods, it appears that cell-associated virions are eventually degraded in the absence of Vpr. However, more studies are needed to conclusively determine what happens to virions in HIV-1-infected MDMs lacking Vpr.

We also found that virions released by MDMs lacking Vpr had, on average, 2- to 3-fold less Env, confirming our observations that Env is targeted by an MDM-specific factor that is counteracted by Vpr. Interestingly, CD4 expression also interferes with Env incorporation into virions produced by transfected 293 cells that lack Nef. Nef expression counteracts this interference and promotes Env incorporation into virions (Lama et al., 1999). The interference mediated by CD4 appears to be analogous to the MDM-specific restriction we describe herein, which is counteracted by Vpr.

Previously identified Vpr-dependent phenotypes observed in permissive cell-line systems depend on an interaction with the Cul4A-DDB1-DCAF1 ubiquitin ligase (Ahn et al., 2010; Belzile et al., 2007; Hrecka et al., 2011; Le Rouzic et al., 2007; Wen et al., 2007), which activates the SLX4-endonuclease complex. Vpr- and DCAF1-dependent activation of the SLX4 endonuclease complex may suppress accumulation of unprocessed HIV-1 DNA intermediates that otherwise induce *IFNα* and *IFNβ* (Laguette et al., 2014). Thus, the macrophage-specific restriction we describe may result from cellular detection of viral infection and activation of an innate immune response. Several lines of evidence presented herein support this model: (1) the Vpr-dependent SLX4 pathway that suppresses innate immune detection of viral infection was active in primary macrophages, (2) DCAF1, a cellular cofactor required for Vpr to activate the SLX4 complex was also required for Vpr-dependent stabilization of Env and efficient release of Env-containing virions in macrophages, (3) Vpr significantly reduced *IFNα* induction in macrophages from three donors at early time points, (4) the magnitude of *IFNα* induction observed in donor macrophages correlated with the magnitude of the Vpr-dependent virion production phenotype detected in macrophages from the same donor at later time points, and (5) addition of IFNα dramatically reduced Env expression and release of virions even in infected macrophage cultures that expressed Vpr. Thus, these data support a model in which Vpr functions to prevent innate immune recognition of infection, *IFN* induction, and the activation of downstream pathways that disrupt Env protein expression and virion release. Whether SLX4 is directly required for *IFN* modulation and whether low amounts of type I IFN induced at the time of initial infection are sufficient for the macrophage restriction will require further experimentation.

Importantly, our results provide strong and convincing evidence that the Vpr-DCAF1 complex is linked to a virological endpoint in a restricted cell type. The observation that Vpr affects a late process in the viral life cycle was initially surprising, because the efficient incorporation of Vpr into virions suggests it acts on a step in the viral life cycle preceding integration (Lu et al., 1995). Indeed, we report here that Vpr causes accelerated turnover of DCAF1 via a proteasomal mechanism as early as 5 hpi and prior to the establishment of productive infection. Thus, Vpr packaged into virions is sufficient to initiate DCAF1-dependent pathways. Interestingly, the proteasome inhibitor, MG132, blocked Vpr-induced early turnover of DCAF1, but did not affect macrophage restriction of Env expression that occurred later, after infection was established. This observation suggests that at least a component of Vpr's capacity to rescue Env expression resulted from an indirect, downstream effect of DCAF1 and Vpr. Whether viral sensing that occurs in the absence of Vpr results in the upregulation of a cellular factor that binds and disrupts Env and Env-containing virions is an interesting possibility that remains to be investigated.

In addition to DCAF1, Vpr also accelerates the turnover of cellular cofactors MUS81, the endonuclease contained within the SLX4 complex that is activated by Vpr to possibly degrade HIV-1 replication intermediates and limit induction of the innate immune response (Laguette et al., 2014), and UNG2, a uracil glycosylase (Priet et al., 2003; Wen et al., 2012). Interestingly, UNG2 may reduce the accumulation of uracilated DNA intermediates in HIV-1-infected primary $CD4^+$ T cells expressing Vpr (Norman et al., 2011). Whether UNG2 and the SLX4 complex work cooperatively to clear HIV-1 intermediates and prevent immune activation in HIV-1-infected primary cell systems is an interesting hypothesis that remains to be investigated.

In summary, we have determined that Vpr increases spread of HIV-1 in MDMs by counteracting an MDM-specific restriction of Env expression that leads to lysosomal degradation of Env and impaired release of Env-containing virions. Notably, this pathway relies upon the expression of the Vpr cofactor DCAF1, and this interaction is necessary for optimal infection of MDMs, a restricted primary cell type. Macrophages represent an important conduit for HIV-1 infection of $CD4^+$ T cells and are infected during the acute phase of HIV-1 infection (Hladik et al., 2007). Thus, these studies provide important insights into how HIV-1 evades the innate immune pathways that would otherwise recognize and restrict viral infection in primary cells that are the targets of HIV-1 in vivo.

EXPERIMENTAL PROCEDURES

Antibodies, cell lines, and viral constructs are described in Supplemental Experimental Procedures.

Cell Culture and Viral Infection

Leukocytes isolated from anonymous donors by apheresis were obtained from New York Blood Center Component Laboratory (Long Island City). Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll density gradient separation as described (Norman et al., 2011). CEMx174 cells were cultured in R10 and infected with HIV-1 by spinoculation as described in Supplemental Experimental Procedures (Salter et al., 1985).

$CD14^+$ monocytes were isolated by positive selection with an EasySep magnetic sorting kit (STEMCELL Technologies). MDMs were obtained by culturing monocytes in R10 (RPMI-1640 with 10% fetal bovine serum [Gibco, Invitrogen], penicillin [10 U/ml], streptomycin [10 μg/ml], L-glutamine [292 μg/ml], M-CSF [50 ng/ml, R&D Systems], and GM-CSF [50 ng/ml, R&D Systems]) for 7–9 days, as described (Lahouassa et al., 2012). MDMs were incubated with HIV-1 for 4 hr, washed with PBS, and cultured in fresh medium, as described (Peeters and Courgnaud, 2002). For IFNα treatment experiments, cells were treated with 500 U/ml recombinant human IFNα (Calbiochem) as described in Figure 7.

Flow Cytometric Analysis

Intracellular staining for Gag CAp24 expression was performed as described previously (Carter et al., 2010).

Western Blotting

MDM WCL Preparation

MDMs infected with HIV-1 were washed with PBS before being lysed in Blue Loading Buffer (Cell Signaling Technology). WCLs were sonicated with a Misonix Sonicator (QSonica) and clarified by centrifugation at 13,000 rpm.

Viral Lysate Preparation

Supernatant from infected cells was passed through a 0.45 μm filter, and virions were pelleted by ultracentrifugation at 25,000 rpm, as described (McCall et al., 2008). The virus-containing pellet was lysed in Blue Loading Buffer and clarified by centrifugation at 13,000 rpm.

Lysates were analyzed by immunoblot, and protein levels were quantified using Adobe Photoshop as described (Norman et al., 2011).

Virion Quantitation

Supernatant containing viral particles was lysed in Triton X lysis buffer (0.05% Tween 20, 0.5% Triton X-100, 0.5% casein in PBS). Antibody to HIV-1 CAp24 antibody (clone 183-H12-5C) was bound to Nunc MaxiSorp plates. Lysed samples were captured for 1–2 hr and incubated with biotinylated antibody to HIV-1CAp24 (clone 31-90-25). 31-90-25 was biotinylated with the EZ-Link Micro Sulfo-NHS-Biotinylation Kit (Pierce). Samples were detected using streptavidin-HRP (Fitzgerald) and 3,3′,5,5′-tetramethylbenzidine substrate, as described (Salmon and Trono, 2007). CAp24 concentrations were measured by comparison to recombinant HIV-1 CAp24 standards (ViroGen). Virion production was normalized for infected cell number by dividing the CAp24 measured by ELISA in supernatant by the number of Gag^+ cells acquired by flow cytometry within a fixed interval of time.

Preparation of $CD4^+$ Lymphocytes

$CD4^+$ lymphocytes were prepared as follows: adherence-depleted PBMCs were depleted of $CD56^+$ cells (EasySep, STEMCELL Technologies) and $CD8^+$ cells (Dynabeads, Sigma) by negative selection. $CD4^+$ lymphocytes were stimulated in R10 and phytohaemagglutinin (PHA; 5 μg/ml). IL-2 (500 IU/ml) was added to the culture 24 hr following PHA stimulation. $CD4^+$ T cells were infected with HIV-1 by spinoculation (2,500 rpm at 25°C for 2–3 hr) 48–72 hr following PHA stimulation, as described (Norman et al., 2011). Infected cells were maintained in R10 and IL-2 until analyzed.

Quantitative PCR

DNA was isolated from 4 to 5 × 10^5 MDMs using the DNeasy Blood & Tissue Kit (Qiagen). HIV-1 DNA and *ACTB* DNA were quantified by quantitative PCR (qPCR) using specific primers as described previously (Clouse et al., 1989; McNamara et al., 2013; Norman et al., 2011). Dilutions of ACH-2 DNA (Clouse et al., 1989; Folks et al., 1989) were used to calculate *gag* copy number and relative DNA input.

Quantitative RT-PCR

RNA was isolated from MDMs using RNeasy Kit (Qiagen) with on-column DNase I digestion. RNA was reverse transcribed using AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies) with 8.5 ng/μl oligo dT and 1.5 ng/μl random nonamers. cDNA was amplified with QuantiTect SYBR Green PCR Kit (Qiagen) on an Applied Biosystems 7300 Real-Time PCR System using commercially available *IFNA1* primers (Prime PCR, qHsaCED0020782, Bio-Rad) or with TaqMan Gene Expression Master Mix with *β-actin* primers and FAM-MGB probes (TaqMan Gene Expression, Hs99999903_m1, Life Technologies) (Applied Biosystems). Reactions were quantified using ABI Sequence Detection software compared to serial dilutions of a single-stranded DNA oligo spanning the *IFNA1* amplicon, or cDNA from mock-treated cells. Calculated copies from the no-RT controls were subtracted from the calculated copies of the cDNA samples, then normalized for input measured by *β-actin*.

Radioimmunoprecipitation Assay

Metabolic labeling of HIV-1-infected MDMs was performed as described (Ono and Freed, 1999). For additional details, see Supplemental Experimental Procedures.

RNA Interference

Short hairpin RNA-mediated silencing of *DCAF1* was performed as previously described (Pertel et al., 2011). Briefly, we spinoculated primary monocytes with VSV-G-pseudotyped $SIV3^+$ for 2 hr with 10 μg/ml polybrene to allow Vpx-dependent downmodulation of SAMHD1. Cells were then incubated overnight in RPMI+10% certified endotoxin-low FBS (Invitrogen) with M-CSF (50 ng/ml, R&D Systems) and GM-CSF (50 ng/ml, R&D Systems) plus 20 μg VSV-G-pseudotyped lentivirus containing an shRNA cassette targeting *luciferase* (control) or *DCAF1*. Following an overnight incubation, the cells were cultured for 3 days in fresh medium before addition of 10 μg/ml puromycin for 3 additional days prior to infection.

Generation of 293T-MDM Heterokaryons

293T cells transfected with p89.6 or p89.6*vpr*$^-$ and paramyxovirus fusion protein expression plasmids (pCAGGS-NDV-HN and pCAGGS-NDV-F, Dr. Trudy Morrison, University of Massachusetts Medical School; McGinnes and Morrison, 2006) were stained with Vybrant DiO (Invitrogen). Uninfected MDMs prepared as described above were stained with Vybrant DiD (Invitrogen). Transfected 293T cells were cultured alone or with MDMs (3:1, 293T:MDM) for 4 days in media containing 2 μM raltegravir and were trypsinized prior to harvest.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.10.014.

AUTHOR CONTRIBUTIONS

M.M., D.R.C., and K.L.C. designed the studies and analyzed the data. M.M. and D.R.C. performed most of the assays. M.M. wrote the first draft of the manuscript with assistance from D.R.C. and V.H.T. V.H.T. performed and analyzed RT-PCR experiments. K.L.C. supervised the research. All authors discussed the results and edited the final manuscript.

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